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INITIAL MICROBIAL DEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS

Article Highlights

- Bacterial PAH degradation was studied using 2,6-dichlorophenol-indophenol assay and GC analysis
- *Rhodococcus* sp. RNP05 degraded 26.0% of pyrene and 89.6% of phenanthrene after 10 days
- *Planomicrobium* sp. RNP01 degraded 5.2% of pyrene and 62.3% of phenanthrene after 10 days
- *Rhodococcus* sp. RNP05 degraded a PAH mixture more efficiently than *Planomicrobium* sp. RNP01
- The bacteria are likely candidates for degradation of highly toxic PAHs in contaminated areas

Abstract

The group of polycyclic aromatic hydrocarbons (PAHs) are very hazardous environmental pollutants because of their mutagenic, carcinogenic and toxic effects on living systems. The aim of this study was to examine and compare the ability and efficiency of selected bacterial isolates obtained from oil-contaminated areas to biodegrade PAHs. The potential of the bacteria to biodegrade various aromatic hydrocarbons was assessed using the 2,6-dichlorophenol-indophenol assay. Further biodegradation of PAHs was monitored by gravimetric and gas-chromatographic analysis. Among the eight bacterial isolates, identified on the basis of 16S rDNA sequences, two isolates, Planomicrobium sp. RNP01 and Rhodococcus sp. RNP05, had the ability to grow on and utilize almost all examined hydrocarbons. Those isolates were further examined for biodegradation of phenanthrene and pyrene, as single substrates, and as a mixture, in vitro for ten days. After three days, both isolates degraded a significant amount phenanthrene, which has a simpler chemical structure than pyrene. Planomicrobium sp.RNP01 commenced biodegradation of pyrene in the PAH mixture only after it had almost completely degraded phenanthrene. The isolated and characterized bacteria, Planomicrobium sp. RNP01 and Rhodococcus sp. RNP05, have shown high bioremediation potential and are likely candidates to be used for degradation of highly toxic PAHs in contaminated areas.

Keywords: PAH biodegradation, Planomicrobium, Rhodococcus, phenanthrene, pyrene.

Polycyclic aromatic hydrocarbons (PAHs) is the common name for a class of several hundred different compounds, which typically contain two to seven condensed benzene rings. These compounds are assimilated into the environment due to intentional combustion of coal, oil products, wood or straw, the discharge of crude oil and petroleum products, as well as during accidental fires [1]. Molecular stability, hydrophobic effects, as well as low solubility of PAH compounds in water are some of the main factors that contribute to their persistence in the environment. Some of these properties are also correlated with the size of the molecule and the number of aromatic rings [2].

Since these compounds pose a major risk to human health and the environment, are toxic and per-

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sistent, and have high potential for exposure to the human population, the majority of them are on the list of compounds regulated by European laws, such as the European Regulation on chemicals - REACH [3], and are also regulated by the US Agency for Environmental Protection (United States Environmental Protection Agency - US EPA).

US EPA specifies 16 PAHs on the list of priority pollutants: naphthalene, acenaphthene, acenaphthylene, anthracene, phenanthrene, fluorene, fluoranthene, pyrene, benzo(*g*,*h*,*i*)perylene, benzo(*a*)anthracene, krisen, benzo(*a*)pyrene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, dibenzo(*a*,*h*)anthracene, and indeno(1,2,3-*cd*)pyrene; the last seven are considered human carcinogens [4].

Under certain conditions, some microorganisms can biodegrade PAHs by using them as a source of energy. This can be an important process if the final effect of this activity is a reduction of environmental pollution. The use of biodegradation processes in remediation, including bioremediation of an environment, is a promising technique for the recovery of contaminated areas due to the relatively low cost and minimal negative impact on the environment. Pyrene and phenanthrene, which contain four and three aromatic rings, respectively, are often used as model compounds for studying the degradation of PAHs [5-7].

In this study, we examined the biodegradation of pyrene and phenanthrene as model compounds for biodegradation of high molecular weight PAHs at the beginning of the biodegradation process, in the initial days of contact of bacterial isolates with the substrates, in order to further develop a bioremediation process for decontaminating soil polluted with these hazardous compounds.

EXPERIMENTAL

Isolation of pure bacterial cultures

Pure bacterial cultures were isolated from a soil sample contaminated by petroleum derivatives from the area of the Pančevo Oil Refinery. The soil was excavated from contaminated area that had been polluted with petroleum derivates from reservoirs for the last ten years. Mineral medium consisted of 0.1% NH_4NO_3 and 0.025% K_2HPO_4 (MM), and containing 2000 ppm of diesel fuel D2 as the sole source of carbon (DM), was inoculated with contaminated soil (20 g/L) and incubated for 28 days in aerobic conditions (in a shaker at 200 rpm, 28 °C) [8]. After incubation, aliquots were cultured on solid DM. After two weeks of growth at 28 °C in aerobic conditions, morphologically different colonies were streaked and cultured three times on solid DM to produce pure cultures. The ability of the isolated bacteria to use hydrocarbons as the sole source of carbon was confirmed by sub-culturing in liquid DM three times [9].

Identification of the bacterial isolates

Bacterial isolates were identified by molecular analysis of 16S rDNA. 16S rDNA genes were amplified by PCR using 27F (50-AGAGTTTGATCMTGGCTCAG-30 [10]) and 1492R primers (50-CGGCTACCTTGTTACGACTT-30 [11]). Taxonomic analysis was conducted using the BLAST program and sequences were deposited in the NCBI GenBank, a public database of genomic and gene sequences (Table 1).

Screening assay for determination of biodegradation potential of isolated bacteria

Bacterial isolates were screened for their ability to biodegrade various hydrocarbons (toluene - TOL, phenanthrene - PHE, pyrene - PYR, dibenzothiophene - DBT) using the modified 2,6-dichlorophenol--indophenol (2,6-DCPIP) assay [12]. Isolated pure bacterial strains were grown individually in liquid DM for 48 h. After incubation, the cell cultures were centrifuged at 6000 rpm at 10 °C for 20 min. The cell pellets were washed twice with sterile saline solution and then suspended in sterile MM to achieve 0.3 density according to the McFarland scale, to produce bacterial suspensions. Polystyrene 24 well macrotiter plate wells contained 1.5 ml of mineral medium, 50 µl of 2,6-DCPIP reagent (150 mg/ml), 150 µl of bacterial suspension and sterilized substrate solution (0.7 ppm of hydrocarbon). Bacterial growth on each hydrocarbon was tested in triplicate. Control wells did not contain added bacterial suspension. Macrotitre plates were incubated for 28 days at 28 °C with rotary stirring at 100 rpm. Color change was recorded as [+] in wells where discoloration occurred, or [-] where wells stayed blue. All assays were performed in triplicate.

Degradation of high molecular weight PAHs

The ability of the bacterial isolates to degrade PAHs was examined using the individual substrates, phenanthrene and pyrene, as well as using a mixture of pyrene:phenanthrene (1:1 mass ratio), at a final concentration of 50 ppm in the MM. MM (100 ml, with pyrene, phenanthrene or pyrene/phenanthrene mixture) was inoculated with 1 ml of bacterial cell suspension (density: 1 McFarland; prepared as described above) in 500 ml Erlenmeyer flasks which were incubated for ten days, at 28 °C with rotary stirring at 100 rpm. Three flasks with identical content were prepared for each bacterium/substrate combination, and

Table 1. Taxonomic identification of the bacterial isolates; G: Genus,Ph: Phylum, C: Class

Strain	Identification	GenBank Accession No.	No. of nucleotides	Sequence aligment, %	Identity[a], %	Nearest phylogenetic neighbour
RNP01	G: *Planomicrobium* sp., Ph: *Firmicutes,* C: *Bacilli*	JN683359	1330	100	97	*P. okeanokoites* IFO 12536 (NR025864)
RNP02	G: *Micrococcus* sp., Ph: *Actinobact.*, C: *Actinobact.*	JN683360	1215	100	99	*M. yunnanensis* YIM 65004 (FJ214355)
RNP03	G: *Staphylococcus* sp., Ph: *Firmicutes,* C: *Bacilli*	JN683361	1327	99	99	*S. warneri* AW 25 (NR025922)
RNP04	G: *Micrococcus* sp., Ph: *Actinobact.,* C:*Actinobact.*	JN683362	1285	100	99	*M. yunnanensis* 9D (JF792072)
RNP05	G:*Rhodococcus* sp., Ph: *Actinobact.,* C: *Actinobact.*	JQ065876	1389	100	99	*R. erythropolis* N11 (NR043535)
RNP06	G:*Staphylococcus* sp., Ph: *Firmicutes,* C: *Bacilli*	JN683363	1321	100	98	*S. pasteuri* ATCC51129 (NR024669)
RNP07	G: *Planococcus* sp., Ph: *Firmicutes,* C: *Bacilli*	JN683364	1205	99	97	*P. salinarum* ISL-16 (FJ765415)
RNP08	G: *Micrococcus* sp., Ph: *Actinobact.,* C: *Actinobact.*	JN683365	1176	100	94	*M. yunnanensis* 17D (JF792083)

[a]The percentage identity with the 16S rDNA sequence of the nearest phylogenetic neighbor

which were sacrificed one by one on days 3, 7 and 10 in order to extract the remaining PAH from the medium.

The remaining PAH was extracted from whole medium three times with n-hexane (50 ml). The organic layer was collected and washed with 50 ml 2% NaCl solution. After dehydration over anhydrous Na_2SO_4, it was concentrated using a vacuum rotary evaporator until the organic solvent was completely removed.

The amount of extracted PAH was determined gravimetrically (the exact mass of the extract was measured after complete evaporation of the solvent) and the extracts were analyzed by gas chromatography.

Gas chromatography analysis of petroleum hydrocarbons

In order to quantify the content of the selected PAH model compounds, all samples were dissolved in the same volume of solvent (1 ml), and each sample was analyzed instrumentally by injecting the same volume of solution (1 μl).

A gas chromatograph (Agilent 4890D) with flame ionisation detector (FID) and HP-5MS column (30 m×0.25 mm, 0.25 μm film thickness) was used for gas chromatography. The carrier gas was hydrogen with a constant flow rate of 1 ml min^{-1}. Injector temperature was constant (250 °C), as was the detector temperature (300 °C). The following temperature ramping was used: initial temperature 100 °C, followed by heating at a rate of 7 °C min^{-1} up to a temperature of 285 °C.

RESULTS AND DISCUSSION

Identification of bacterial isolates

Eight bacterial isolates which could use petroleum type hydrocarbons as their only carbon source were isolated on MM supplemented with diesel fuel, and identified. The bacterial isolates were members of the genera *Planomicrobium*, *Planococcus*, *Staphylococcus*, *Micrococcus* and *Rhodococcus* (Table 1).

Biodegradation potential of bacterial isolates using the 2,6-DCPIP screening assay

2,6-DCPIP is redox indicator used to detect oxidation of NADH to NAD^+, which occurs, among other processes, during bacterial degradation of hydrocarbons. The assay detects the color change of 2,6-DCPIP from the blue oxidized form to the colorless, reduced form. In this study, the biodegradation potential of the bacterial isolates after 28 days growth on hydrocarbons was examined using the 2,6-DCPIP assay. The bacterial isolates showed differences in their ability to biodegrade the hydrocarbon substrates, shown in Table 2.

The results showed that the 2,6-DCPIP assay has sufficient sensitivity to detect primary oxidation of hydrocarbons after the first four weeks of the biodegradation process and can be used as a quick screening method for large numbers of potential hydrocarbon degraders. This assay has been successfully implemented in other studies of indigenous microorganisms that may contribute to the cleaning of soil contaminated with petroleum pollutants [13]. Thenmozhi

et al. used this assay to evaluate the ability of *Serratia*, *Pseudomonas* and *Bacillus* isolated from engine oil to degrade polycyclic aromatic compounds; analysis of the plasmid DNA profile of those bacteria confirmed they possessed genes which contribute to the degradation of hydrocarbons [14].

Table 2. Bacterial growth on hydrocarbons indicated using the 2,6-DCPIP assay

Bacterial isolate	TOL	PHE	PYR	DBT
Planomicrobium sp. RNP01	-	+	+	+
Micrococcus sp. RNP02	-	-	-	-
Staphylococcus sp. RNP03	-	-	+	+
Micrococcus sp. RNP04	-	-	-	+
Rhodococcus sp. RNP05	+	+	+	+
Staphylococcus sp. RNP06	-	-	-	+
Planococcus sp. RNP07	-	+	-	+
Micrococcus sp. RNP08	-	-	+	+

In the current study, almost all isolates were able to degrade DBT, while toluene was degraded by only one microorganism. Two isolates, *Planomicrobium* sp. RNP01 and *Rhodococcus* sp. RNP05, showed the highest potential for degradation of the examined hydrocarbons.

Initial biodegradation of high molecular weight PAHs

Based on the 2,6-DCPIP assay, the two most active isolates were selected for detailed examination of biodegradation of high molecular weight PAH compounds using gravimetric and gas chromatographic analysis.

Based on the results presented in Table 3, both bacterial isolates degraded phenathrene faster than pyrene. *Rhodococcus* sp. RNP05 showed greater efficacy in degradation of both PAH model compounds compared to *Planomicrobium* sp. RNP01, which is consistent with the results of the 2,6-DCPIP assay.

According to gravimetric analysis, *Rhodococcus* sp. RNP05 achieved a maximum degradation rate of phenanthrene by day 3 (5.1 ppm/day), and by day 10, the rate had decreased to 1.1 ppm/day. In contrast, this bacterium achieved its maximum rate of pyrene degradation (2.4 ppm/day) on day 10. *Planomicrobium* sp. RNP01 produced its maximum degradation rate of both examined substrates on day 7, 1.8 ppm/day for pyrene and 4.7 ppm/day for phenanthrene. The preference to degrade phenanthrene was common to both isolates, which was expected, having in mind that phenanthrene has a simpler chemical structure than pyrene. These results are consistent with the results of Bidaud and Tran-Minh [15] and McKew *et al*. [16], which showed that the rate of biodegradation of PAH compounds decreases with increasing number of condensed rings.

Table 3. Percentage of high molecular weight PAHs biodegraded over 10 days calculated by GC analysis based on relative decrease of peak areas

Day of cultivation	Isolate	
	Planomicrobium sp. RNP01	*Rhodococcus* sp. RNP05
	PYR biodegradation	
3	2.0	10.2
7	3.7	17.9
10	5.2	26.0
	PHE biodegradation	
3	3.6	30.6
7	49.8	71.7
10	62.3	89.6
	PYR+PHE biodegradation	
3	13.8	17.6
7	33.93	64.1
10	67.53	74.4

Results of biodegradation of the mixture of pyrene and phenathrene revealed that *Rhodococcus* sp. RNP05 was a much more efficient degrader of the PAH mixture than *Planomicrobium* sp. RNP01. By day 7, *Rhodococcus* sp. RNP05 showed significant biodegradation activity, and utilized the mixed PAH substrate at the rate of 5 ppm/day, in contrast to *Planomicrobium* sp. RNP01, which produced a maximum degradation rate of 2.3 ppm/day on day 3. The difference in the amount of degraded substrate increased in favor of *Rhodococcus* sp. RNP05 up to day 10, when this isolate had degraded more than three quarters of the available substrate, while *Planomicrobium* sp. RNP01 had degraded only half of the available substrate in this time. However, these results revealed that *Planomicrobium* sp. RNP01 can produce similar biodegradation potential as *Rhodococcus* sp. RNP05, but needs more time to adapt to the substrate.

Gas chromatographic analysis indicated that both bacterial isolates preferably degraded phenanthrene, which has a simpler chemical structure compared to pyrene (Figure 1). Noticeably, *Planomicrobium* sp. RNP01 began biodegradation of pyrene only after it had almost completely degraded the available phenanthrene (Figure 1).

The biodegradation rate depends on the concentration of substrates, and ability of the bacteria to use a specific substrate as an energy source. It can be assumed that at the highest degradation rate, bacteria grow exponentially due to their metabolic path-

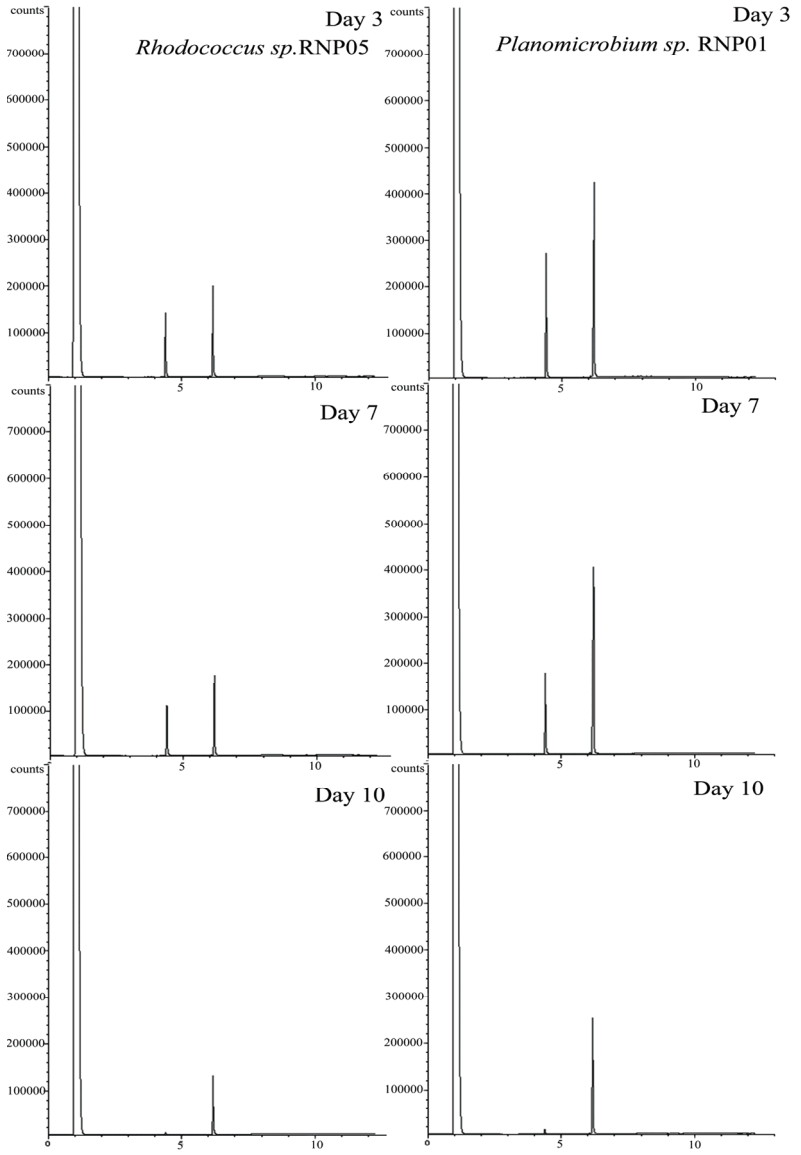

Figure 1. Gas chromatographic analysis of biodegradation of the mixture of high molecular weight PAHs, pyrene and phenathrene.

ways for degradation being already activated and their having available sufficiently high concentrations of substrate; this saturates the bacterial system for uptake of that substrate. When consumption of PAHs becomes greater than their dissolution rate in the medium, the concentration of PAH decreases, as does the bacterial growth rate. The exponential phase of bacterial growth is short because the metabolic demands of the growing number of cells quickly exceed the dissolution rate of PAH [17-20].

In general, petroleum hydrocarbon biodegradation is usually a slow process due to the hydrophobic nature of the contaminants, which causes consequent bioavailability limitations. Measuring the success of bioremediation of oil contaminated sites is based on several parameters, among which is the degradation of PAHs. Bacteria capable of degrading PAHs have been reported previously [21-23].

The genus *Rhodococcus* exhibits a diverse range of metabolic activities, including the degradation of various aromatic and aliphatic hydrocarbons. They can catabolize short- and long-chain alkanes, aromatic (halogenated and nitro-substituted), heterocyclic and polycyclic aromatic compounds. This catabolic diversity was recognized during the 1990s, and this genus soon came to be regarded as one of the most promising groups of organisms for the biodegradation of compounds that cannot be easily transformed by other organisms [24,25]. *Rhodococcus* sp. UW1 was shown to mineralise 72% of the pyrene within two weeks [26]. Pizzul *et al.* reported *Rhodococcus wratislaviensis*, which degraded >90% of

phenanthrene and pyrene after 49 days [27]. As well, *Rhodococcus opacus* from mangrove sediments was found to degrade about 70% of phenanthrene and 20% of pyrene within a 10 day incubation period [28].

Planomicrobium sp. was first described in 2005, and was isolated from coastal sediments of China [29]. After that, many authors reported the occurrence of *Planomicrobium* sp. as a hydrocarbon-degrading bacterium isolated from oil contaminated areas as well as from phyllospheres of various plant species [30-32]. *Planomicrobium chinense* DX-12 completely mineralized diesel oil within 96h and can use mono-aromatic compounds such as bezene, toluene and xylene as source of C for growth [33]. Bacteria such as *Planomicrobium alkanoclasticum* strain MAE2 selectively degrade linear and branched alkanes, but cannot degrade aromatic hydrocarbons [34].

However, some other bacterial cultures required other PAHs, peptone or yeast extract to stimulate pyrene degradation [21]. *Mycobacterium* sp. strain 1B was able to utilize phenanthrene, pyrene, and fluoranthene as sole carbon sources for growth. However, when benzo[*a*]pyrene was used as the sole carbon source, no benzo[*a*]pyrene removal or cell growth was observed [23]. In contrast, our bacterial isolates were able to utilize all examined PAHs for growth in the absence of other PAHs.

CONCLUSION

Biological degradation and removal of harmful substances from the environment by microorganisms is a current trend in the decontamination of polluted areas. As a proper approach to the bioremediation of polluted areas, there is a need to select and isolate microorganisms from the contaminated environment and to examine their effectiveness in terms of degradation of the substrate, with the ultimate goal of using those isolates in environmental purification processes.

The current results show that in highly contaminated areas, a variety of bacterial isolates capable of rapid adaptation for the efficient biodegradation of contaminating PAH compounds can be found. Among the bacterial isolates detected, *Planomicrobium* sp. RNP01 and *Rhodococcus* sp. RNP05 proved to be particularly efficient degraders of PAH compounds. However, residual levels of PAHs post-remediation and kinetics of degradation would depend not only on the type of microorganism but also on the structure of the PAH molecules in the contaminated environment.

The results strongly indicate that the two isolated and characterized bacteria, *Planomicrobium* sp. RNP01 and *Rhodococcus* sp. RNP05, have high bioremediation potential and are likely candidates to be used for degradation of highly toxic PAHs in contaminated areas.

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NAUČNI RAD

INICIJALNA MIKROBIOLOŠKA DEGRADACIJA POLICIKLIČNIH AROMATIČNIH UGLJOVODONIKA

Policiklični aromatični ugljovodonici (polycyclic aromatic hydrocarbons - PAHs) spadaju u grupu jedinjenja koja predstavljaju veoma opasne zagadjivače životne sredine zbog svojih mutagenih, kancerogenih i toksičnih efekata na živi svet. Cilj ovog rada bio je da se ispita i uporedi sposobnost i efikasnost bakterijskih izolata dobijenih iz oblasti zagađenih naftim derivatima u razgradnji PAHs. Bakterijski potencijal za biološku razgradnju različitih aromatičnih ugljovodonika određen je pomoću 2,6-dihlorofenol-indofenol testa. Biodegradacija PAHs analizirana je gravimetrijskom i gasno-hromatografskom tehnikom. Od osam bakterijskih izolata selektovana su dva izolata, identifikovana na osnovu 16S rDNK kao Planomicrobium sp. RNP01 i Rhodococcus sp. RNP05, koji imaju sposobnost da rastu i iskorišćavaju skoro sve testirane ugljovodonične supstrate. Selektovani bakterijski izolati korišćeni su za biodegradaciju fenantrena i pirena, kao pojedinačnih supstrata, ali i kao smeše, deset dana u in vitro uslovima. Nako tri dana od početka eksperimenta, oba bakterijska soja razgrađuju značajnu količinu fenantrena, koji ima jednostavniju hemijsku strukturu od pirena. Planomicrobium sp.RNP01 započinje biodegradaciju pirena u smeši tek nakon što je skoro u potpunosti razgradio fenantren. Izolovani i okarakterisani bakterijski sojevi, Planomicrobium sp. RNP01 i Rhodococcus sp. RNP05, pokazali su visok bioremedijacioni potencijal i mogu biti kandidati za razgradnju veoma toksičnih PAHs na zagađenim područjima.

Ključne reči: PAH biodegradacija, Planomicrobium, Rhodococcus, fenantren, piren.